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Polyethylene particles of a 'critical size' are necessary for the induction of cytokines by macrophages in vitro

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Abstract

Particulate wear debris from total hip prosthetic components can stimulate macrophages to produce mediators of osteolysis which may cause aseptic implant loosening. This study evaluated the in vitro response of murine peritoneal macrophages to polyethylene particles of difinitive size distributions at varying volume doses. Ceridust 3615 polyethylene particles with a mean size of 0.21, 0.49, 4.3 and 7.2 μm and GUR1120 polyethylene resin with a mean size of 88 μm were co-cultured with C3H murine peritoneal macrophages at volume $(\mu m)^3$ to cell number ratios of 100:1, 10:1, 1:1 and 0.1:1. The secretion of IL-6, IL-1β and TNF-α was determined by ELISA. Significantly elevated levels of TNF-α and IL-1β were determined at 100:1 ratios when the macrophages were challenged with particles with a mean size of 0.49, 4.3 and 7.2 μm, and at 10:1 ratios for particles with a mean size of 0.49 and 4.3 μm. IL-6 production was significantly elevated at 100:1 ratios for mean particle sizes of 0.49 and 4.3 μm. Particles outside this range produced considerably less cytokine suggesting that both the size and volume (or number) of polyethylene particles are critical factors in macrophage activation. Therefore particles in the phagocytosable size range of 0.3–10 μm appear to be the most biologically active.

1. Introduction

Ultra high molecular weight polyethylene (UHMWPE) wear debris generated at the articulating surfaces of total artificial hip joints is believed to play an important role in periprosthetic osteolysis and ultimately the aseptic loosening of hip replacements [1, 2]. Histological studies of explanted tissue from patients undergoing revision operations have shown a periprosthetic pseudo-membrane rich in macrophages and multinucleated giant cells that are associated with polyethylene particles [3, 4]. It is believed that macrophages actively phagocytose wear debris at the implant–bone interface and release various proteolytic enzymes and osteolytic mediators such as interleukin 1β (IL-1β), interleukin-6 (IL-6), tumour necrosis factor α (TNF-α) and prostaglandin E_2 (PGE_2) causing inflammation and osteoclastic bone resorption [2–11]. The composition, number, size, surface area, shape and volume of wear debris are all thought to influence this macrophage-mediated process [7, 12, 13]. Previous in vitro cell studies have utilised commercially available particles that differ greatly in their size distributions and shape compared to the debris generated clinically. These factors may affect the biological response by macrophages. Studies of polyethylene particles from digested explanted tissue by scanning electron microscopy have revealed that the vast majority of particles are submicron in size with some larger platelets and large shards of debris [14, 15]. It is often suggested that the submicron particles are the most biologically active, however, it was found in a study by Shanbhag et al. [7] that titanium and polystyrene particles less than 0.5 μm in size did not elicit as great a response as particles above 0.5 μm. Unfortunately, there are very few studies that have investigated the role that polyethylene particles play in the osteolytic processes leading to aseptic loosening as it is inherently difficult to work with polyethylene in in vitro culture systems. Some previous studies have

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failed to take into account the fact that polyethylene particles float in culture medium, which limits the macrophages ability to phagocytose them. The aim of this study was to determine the response of macrophages to polyethylene particles of various sizes and doses. The macrophage–particle interactions have been optimised by holding the polyethylene particles superficially in agarose gels and culturing the primary macrophages on top of the particle–agarose gel. The polyethylene particles were separated and tested within definitive size ranges in order to determine the 'critical' size range for macrophage activation.

2. Materials and methods

2.1. Particle isolation and characterisation

Ceridust 3615 polyethylene powder (Hoescht, Germany) was suspended in RPMI 1640 culture medium (Gibco, UK) and sonicated for 1 h. The suspension was then sequentially filtered through 10, 1, 0.6, 0.4, 0.2 and 0.1 μm pore size cyclopore polycarbonate membranes (Whatman Ltd. UK) and the mass of debris on each membrane was calculated. The size distribution of the particles on the 10, 1, 0.4 and 0.1 μm membranes was then determined by scanning electron microscopy (SEM; Joel JSM T20; Joel; Tokyo, Japan) and image analysis (microscale TC Image Analysis system, Digithurst, UK) by measuring the greatest length and breadth of the particles resulting in mean particle sizes ± standard deviations of 7.2 ± 3.15 μm, 4.3 ± 1.89 μm, 0.49 μm $\pm$ 0.11 μm and 0.21 ± 0.069 μm (Fig. 1). GUR1120 UHMWPE resin (Hoescht, Germany), with a mean size of 88 μm ± standard deviation of 29 μm was used to represent the large shards of debris generated clinically. The particles were then sterilised by gamma irradiation (25 Gy, Caesium^{137} source).

The particle volumes to be added to macrophages in culture were determined by mass. Since 1 $(\mu m)^3$ of polyethylene powder had an equivalent mass of 1×10^{-9} mg, the mass of polyethlene within a given size range could be theoretically converted into the volume of particles and hence the particle volume $(\mu m)^3$: macrophage cell number ratios could be determined.

Each particle size fraction was resuspended in macrophage culture medium which consisted of RPMI 1640 without L-glutamine (Life Technologies, UK), 10% (v/v) heat-inactivated foetal calf serum (Life Technologies, UK), 100 U ml^{-1} penicillin (ICN, UK), 100 μg ml^{-1} streptomycin (ICN, UK), 2 mM L-glutamine (Life Technologies, UK), 15 mM *N*-(2-hydroxyethyl) piperazine-*N'*-2-ethanesulphonic acid (HEPES Life Technologies, UK) and 0.05 mM 2-mercaptoethanol (Life Technologies, UK), by sonication of the appropriate particle laden filter in the macrophage culture medium for 1 h. To ensure

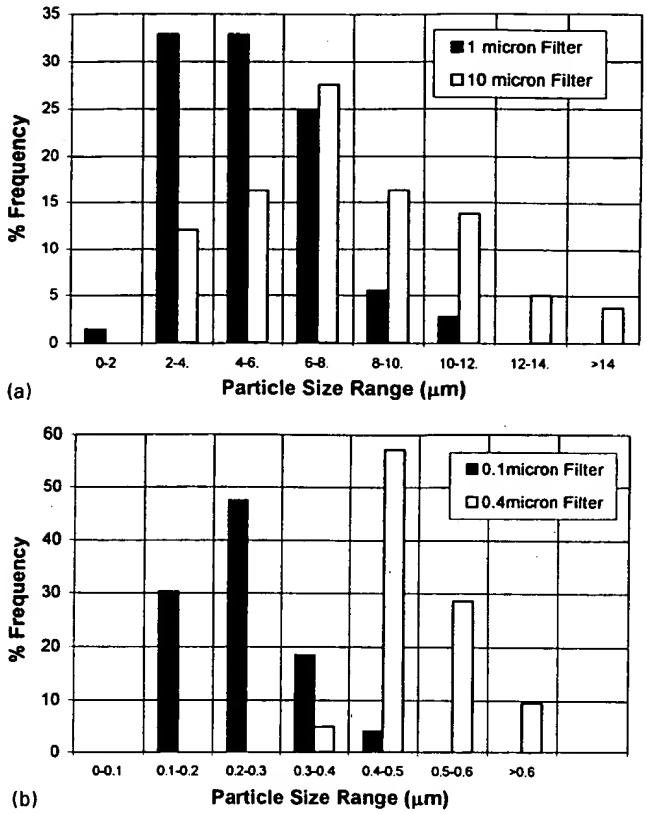

Fig. 1. (a) The size distributions of ceridust 3615 polyethylene particles on 1 and 10 micron cyclopore polycarbonate filters. (b) The size distributions of ceridust 3615 polyethylene particles on 0.1 and 0.4 micron cyclopore polycarbonate filters.

that all the particles had been retrieved the filters were subsequently re-weighed and re-analysed by SEM.

2.2. Macrophage isolation

C3H/Hej mice (40; Biomedical Services, University of Leeds, UK) were killed by CO_2 asphyxiation and subjected to peritoneal lavage by injecting the peritoneal cavity with 5 ml of sterile sodium chloride solution (Phoenix pharmaceuticals Ltd., UK). The peritoneal lavage fluid was pooled and centrifuged at 55 *g* for 10 min. The cells were resuspended in macrophage culture medium (as above) and incubated overnight in bacteriological grade polystyrene petri dishes in 5% (v/v) CO_2 in air at 37°C. After the overnight incubation, non-adherent cells were removed by washing with Hank's balanced salt solution (HBSS) without calcium and magnesium (Life Technologies, UK), leaving the adherent macrophages, which were retrieved using a non-enzymatic cell dissociation solution (designed for the gentle removal of cells and preservation of cellular proteins; Sigma, UK). The macrophages were then centrifuged at 55 *g* for 10 min

and resuspended in macrophage culture medium at 5×10^5 cells ml^{-1}. Macrophages isolated using these methods are resident mature cells.

2.3. Co-culture of macrophages with particles

A 1% (v/v) agarose (Ultra pure, low melting point; Life Technologies, UK) solution was prepared in macrophage culture medium and sterilised by autoclaving at 115°C for 15 min. One volume of the 1% (v/v) agarose solution was mixed with two volumes of each of the particle solutions to give particle volume (μm^3) to macrophage cell number ratios of 100:1, 10:1, 1:1 and 0.1:1. The mixtures were then added to 48 well plates in 200 µl volumes in triplicate and centrifuged at 800 *g*. This resulted in a superficial layer of polyethylene particles [16]. Peritoneal macrophages (1 ml; 5×10^5 cells) were then added to the agarose/particle gels. Culture medium without particles mixed with 1% (v/v) agarose, and lipopolysaccharide (LPS; Sigma, UK) at 1 µg ml^{-1} was used as a positive control and culture medium without particles mixed with 1% (v/v) agarose was the negative control. The tissue culture plates were then incubated in 5% (v/v) CO_2 in air at 37°C for 24 h. After incubation the culture supernatants were harvested and stored at − 70°C for cytokine determinations.

2.4. MTT Assay for cell viability

Following the aspiration of the culture supernatants, the viability of the macrophages was assessed by (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; thiazolyl blue) (MTT; Sigma, UK) conversion. The wells were replenished with 500 µl of culture medium and 25 µl of MTT (5 mg ml^{-1}; in phosphate buffered saline; PBS; pH 7.4) was added to each well. Tissue culture plates were incubated in 5% (v/v) CO_2 in air at 37°C for 4 h, 250 µl of 10% (w/v) lauryl sulfate (SDS; Sigma, UK) in HCl was added to each well and plates incubated for a further 24 h at 37°C. Following incubation, 100 µl of culture medium was aspirated from each well and the optical density was measured at 570 nm. Results are expressed as the mean optical density ± 95% confidence limits.

2.5. Cytokine assays

Levels of IL-6, IL-1β and TNFα were determined by enzyme-linked immunosorbent assays (ELISA). Murine IL-6 was measured using an IL-6 'minikit' (Endogen), murine IL-1β was determined using a pre-coated ELISA kit (Endogen) and murine TNF-α was assayed using a duoset ELISA kit (Genzyme). Levels of IL-6, IL-1β and TNF-α were determined in ng ml^{-1}. Results are expressed in units of specific activity ± 95% confidence limits where specific activity is the quantity of cytokine (ng ml^{-1}) divided by the optical density at 570 nm from the MTT assay.

2.5. Statistical analysis

The results presented are representative of a single experiment in which three replicate cultures of the cells were stimulated with each of the different sizes of particles. Data was analysed by one way analysis of variance (ANOVA). Individual differences between means of particle-stimulated and LPS stimulated cells compared to the negative control were determined by calculating the minimum significant difference (MSD; $P < 0.01$, $n = 3$) using the T-method.

3. Results

3.1. Cell viability

The mean optical densities obtained using the MTT assay were comparable for all the treatment groups and were not significantly different from the negative control indicating that the polyethylene particles did not affect the macrophage cell viability (Fig. 2).

3.2. Interleukin-6

Cultured C3H murine peritoneal macrophages (5×10^5 cells ml^{-1}, 24 h) in the absence of particles secreted 0.44 ± 0.6 units of IL-6 specific activity. Compared to this non-stimulated control group the cells cultured with 0.49 and 4.3 µm particles at particle volume $(\mu m)^3$: macrophage cell number ratios of 100:1 produced 2.41 ± 0.36 and 3.15 ± 0.68 units of IL-6 specific activity respectively ($P < 0.01$, $n = 3$). LPS stimulated cells produced 3.32 ± 0.55 units of IL-6 specific activity ($P < 0.01$, $n = 3$). There was no significant increase in IL-6 secretion for any of the other treatment groups (Fig. 3).

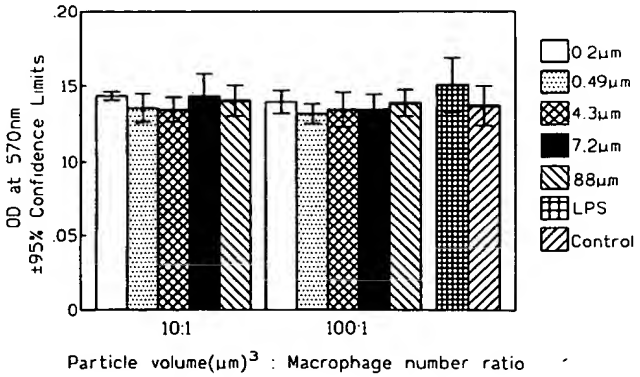

Fig. 2. MTT viability of macrophages stimulated with polyethylene particles of varying sizes and volumes.

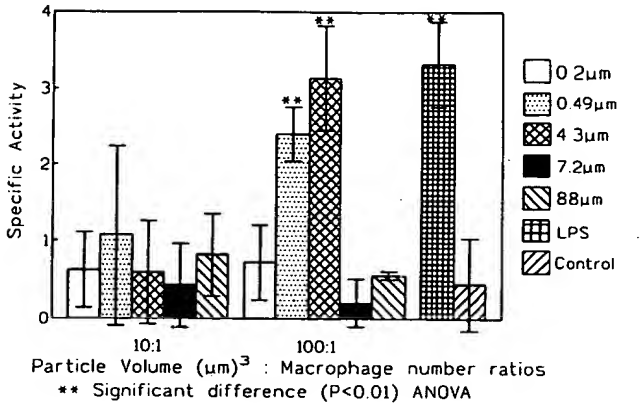

Fig. 3. IL-6 production by peritoneal macrophages stimulated by different size/volumes of polyethylene particles ($P < 0.01$; $n = 3$).

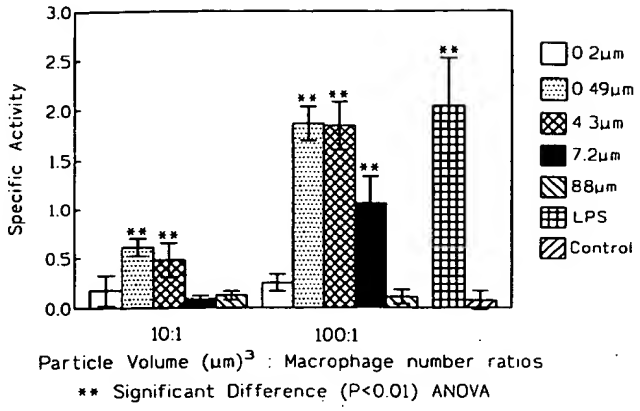

Fig. 4. IL-1β production by peritoneal macrophages stimulated by different size/volumes of polyethylene particles ($P < 0.01$; $n = 3$).

3.3. *Interleukin-1β*

The production of IL-1β by unstimulated C3H murine peritoneal macrophages (5×10^5 cells ml^{-1}, 24 h) was 0.08 ± 0.09 units of IL-1β specific activity. Particles in the size groupings of 0.49, 4.3 and 7.2 µm at particle volume (µm)^3:macrophage cell number ratios of 100:1 produced 1.87 ± 0.17, 1.85 ± 0.242 and 1.065 ± 0.279 units of IL-1β specific activity, respectively ($P < 0.01$, $n = 3$), in comparison to the negative control. At the 10:1 ratio only the 0.49 and 4.3 µm particles stimulated the macrophages to produce elevated levels of IL-1β at 0.61 ± 0.09 and 0.48 ± 0.174 units of IL-1β specific activity ($P < 0.01$, $n = 3$) and LPS stimulated the cells to produce 2.044 ± 0.478 units of IL-1β specific activity ($P < 0.01$, $n = 3$). There was no significant increase in IL-1β secretion for any of the other treatment groups (Fig. 4).

3.4. *TNF-α*

Peritoneal macrophages cultured with culture medium alone secreted 1.14 ± 1.9 units of TNF-α specific activity. Polyethylene particles with a mean size of 0.49, 4.3 and 7.2 µm particles at 100:1 ratios produced 33.3 ± 4.34, 35.58 ± 2.65 and 16.46 ± 10.3 units of TNF-α specific activity respectively ($P < 0.01$, $n = 3$). The 0.49 and 4.3 µm particles at 10:1 ratios also stimulated the cells to produce 38.1 ± 0.3 and 15.58 ± 10.62 units of TNF-α specific activity ($P < 0.01$, $n = 3$) and the positive control stimulated cells to produce 34.73 ± 7.58 units of TNF-α specific activity ($P < 0.01$, $n = 3$). Particles outside these treatment groups did not elicit the release of significantly elevated levels of TNF-α (Fig. 5).

3.5. *Repeatability*

Experiments were repeated using the same particle size distributions at ratios of 10 and 100 µm^3 to one macrophage and the results showed the same relative trend in cytokine production although the absolute levels varied.

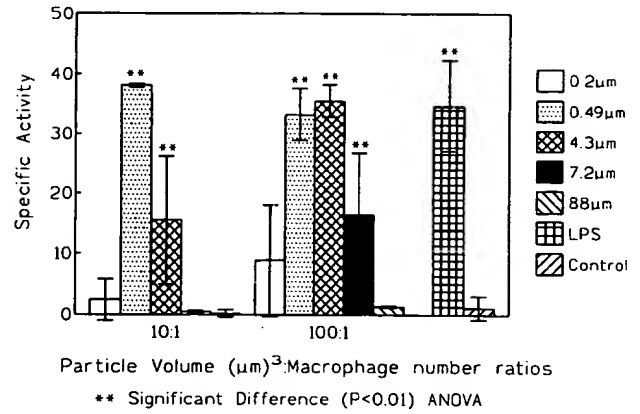

Fig. 5. TNF-α production by peritoneal macrophages stimulated by different size/volumes of polyethylene particles ($P < 0.01$; $n = 3$).

4. Discussion

One of the major problems in carrying out studies of this nature is the spontaneous activation of macrophages during isolation and culture. This is reflected in the levels of cytokines generated in the controls without particles or LPS. During preliminary experiments extensive optimisation of the parameters, i.e. type of agarose, use of non-enzymatic cell dissociation solution, macrophage seeding densities and duration of co-culture was carried out. Nevertheless, variation in the absolute levels of cytokines produced from experiment to experiment were apparent. However, the trend was repeatable. The percentage of agarose used in the culture system was optimised to allow the macrophages to enter the gel to phagocytose the particles. This was validated by light microscopy.

It has been demonstrated in this study that polyethylene particles can stimulate primary macrophages to produce elevated levels of the osteolytic cytokines IL-6, IL-1β and TNF-α in vitro. The results demonstrate that the volume and size of particles are critical factors in macrophage activation, with the most biologically active particles in the phagocytosable size range of 0.3–10 μm. The most important observation of this study was that both the largest (88 ± 29 μm) and the smallest (0.21 ± 0.07 μm) particles tested failed to stimulate the macrophages to produce IL-1β, IL-6 and TNF-α significantly above control levels. The fact that the larger polyethlene particles did not activate the macrophages may be explained by the macrophages inability to phagocytose the particles since they were to large. The failure of the very small polyethylene particles (0.21 ± 0.069 μm) to stimulate the macrophages to produce the osteolytic cytokines is of fundamental importance to the study of wear debris induced osteolysis since submicron particles are generally perceived to be the most biologically active. These results support the work of Shanbhag et al. [7], who observed that the response of macrophages to titanium and polystyrene particles (0.15 and 0.45 μm) was less than for larger particles (1.76 μm). Shanbhag et al., proposed that this effect could be due to the nature in which the macrophage internalizes different size particles, with particles below 0.5 μm in size being taken up with extracellular fluids in a constitutive manner through pinocytosis as opposed to the active phagocytosis of particles greater than 0.5 μm. This study suggests that the 'critical' size range for macrophage activation by polyethylene particles may well extend below than 0.5 μm, since particles with a mean size of 0.49 μm stimulated high levels of IL-6, IL-1β and TNF-α.

Horowitz et al. [18], reported that the principle mediator released by macrophages cultured with polyethylene particles were TNF-α and PGE_2, and did not detect the release of IL-6 and IL-1β, This study has shown that TNF-α is produced at levels over ten-fold higher than IL-6 and IL-1β which may explain the results of Horowitz et al. [18]. In addition TNF-α production was maximal at the particle volume $(\mu m)^3$: macrophage number ratio of 10: 1 whereas maximal IL-6 and IL-1β secretion was observed at the 100: 1 ratios. Horowitz et al. [18] did not detect IL-6 in their study unless osteoblasts were added to the culture system and IL-1β was never detected. The present study demonstrates that the production of IL-6 and IL-1β by macrophages can occur independently of the presence of osteoblasts which is in agreement with a study by Shanbhag et al. [17], who detected IL-1β, IL-1α, IL-6 and PGE_2 production by human monocytes stimulated with fabricated and retrieved polyethylene particles. Shirata et al. [19], demonstrated using IC-21 cells and an inverted tissue culture system that HDPE particles stimulated a significant release of IL-1β. Voronov et al. [20], used a collagen gel system to co-culture IC-21 macrophages with UHMWPE particles (18–20 μm) and HDPE particles (4–10 μm). IL-1β and IL-6 secretion were significant at $P < 0.05$ and $P < 0.1$, respectively. TNF-α secretion was not significant in this system compared with other in vitro studies. The authors stated that this could be explained by the murine cell line they were using and the size of the particles, which were not representative of the sub micron debris found clinically.

Macrophage derived TNF-α, IL-6 and IL-1β are all implicated in the processes leading to bone resorption around total joint replacements, but their precise roles are still under scrutiny [2]. Histological studies of the cytokines present in the retrieved pseudo-membrane from patients with failed prostheses have shown the presence of IL-6, IL-1β and TNF-α [4, 5, 21]. IL-6 is believed to stimulate osteoclast formation and bone resorption. This is supported by the fact that osteoclasts from patients with Paget's disease and giant cell tumor have IL-6 receptors on their cell membrane [22]. IL-1β has been implicated in stimulating the proliferation and maturation of progenitor cells into osteoclasts and through the stimulation of osteoblasts the osteoclasts mature into multinucleated bone resorbing cells [2]. TNF-α also causes the proliferation of osteoclast progenitors and through osteoblasts activates multinucleated osteoclasts to resorb bone [2].

This study suggests that TNF-α maybe one of the principle cytokines to be produced in particle-induced osteolysis. Algan et al. [23], identified TNF-α as the major mediator produced by macrophages exposed to polymethylmethacrylate (PMMA) particulate debris. They observed that bone resorption was inhibited when anti-TNF-α antibody was added to their culture system and that PGE_2 production was partially suppressed. Consequently they hypothesized that PMMA particle-induced osteolysis appears to be initiated by the production of TNF-α by the macrophage which in turn stimulates osteoblasts to produce PGE_2 resulting in osteoclastic bone resorption.

In this study we have demonstrated that the size and volume of polyethylene particles phagocytosed by macrophages are critical factors for osteolytic cytokine secretion. The most biologically active polyethylene particles are in the phagocytosable size range 0.3–10 μm. Polyethylene particles outside this size range did not stimulate macrophages to produce significantly elevated levels of IL-6, IL-1β and TNF-α in comparison with unstimulated controls.

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